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Molecular Determinants of pH Sensitivity of the Type IIa Na/P_i Cotransporter*

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Type II Na/P_i cotransporters play key roles in epithelial P_i transport and thereby contribute to overall P_i homeostasis. Renal proximal tubular brush border membrane expresses the IIa isoform, whereas the IIb isoform is preferentially expressed in small intestinal brush border membrane of mammals. IIa and IIb proteins are predicted to contain eight transmembrane domains with the N- and C-terminal tails facing the cytoplasm. They differ in their pH dependences: the activity of IIa increases at higher pH, whereas the IIb shows no or a slightly opposite pH dependence. To determine the structural domains responsible for the difference in pH sensitivity, mouse IIa and IIb chimeras were constructed, and their pH dependence was characterized. A region between the fourth and fifth transmembrane domains was required for conferring pH sensitivity to the IIa-mediated Na/P_i cotransport. Sequence comparison (IIa *versus* IIb) of the third extracellular loops revealed a stretch of three charged amino acids in IIa (REK) replaced by uncharged residues in IIb (GNT). Introduction of the uncharged GNT sequence (by REK) in IIa abolished its pH dependence, whereas introduction of the charged REK stretch in IIb (by GNT) led to a pH dependence similar to IIa. These findings suggest that charged residues within the third extracellular loop are involved in the pH sensitivity of IIa Na/P_i cotransporter.

The kidney and the small intestine are involved in maintaining overall phosphate (P_i) homeostasis. Renal tubular P_i reabsorption contributes to "acute" and "chronic" regulatory control, whereas only slow "adaptive" changes occur in small intestinal P_i absorption (1–4). Na/P_i cotransporters located in renal proximal tubular and small intestinal brush border membrane are the rate-limiting and physiologically controlled steps (4–6). Recently they have been structurally identified as type II Na/P_i cotransporters (7–11): the proximal tubule expresses the type IIa isoform (7, 12), whereas the type IIb isoform in mammals is preferentially expressed in small intestine and in other tissues such as lung, colon, liver, and testis but not in kidney (11).

Mouse IIa and IIb cotransporters show an overall identity of 57%, which increases to 75% in the predicted transmembrane domains (11). Most of the differences are found in the N- and C-terminal regions (11). A topology model of both cotransporters, based on hydropathy analysis and epitope tagging, predicts eight transmembrane segments, with the N- and C-terminal tails facing the cytoplasm and a large hydrophilic loop between the third and fourth transmembrane domains (7, 11, 13). This large loop is glycosylated (14).

The kinetic properties of mouse IIa and IIb cotransporters have been characterized after expression in *Xenopus laevis* oocytes. There are some differences in the affinities for the substrates, Na^+ and P_i (11). Moreover, IIa and IIb show different pH dependence of both $^{32}P_i$ uptake and P_i-induced electrogenic responses (11, 15). For the IIa, a decrease in external pH results in a significant decrease in transport activity (up to 80%) (7, 15), whereas IIb shows no or a slightly opposite pH dependence (11).

The characteristics of the type IIa cotransporter determine, to a large extent, Na^+-dependent P_i reabsorption at the proximal tubular brush border membrane (16, 17). The pH dependence of this activity has been extensively characterized by vesicle uptake studies (18, 19) and by electrophysiology applied to the IIa cotransporter expressed in *X. laevis* oocytes (7, 15, 20, 21). These data indicate that the pH sensitivity of renal brush border Na/P_i cotransport (IIa-mediated) is not explained by a titration of divalent P_i, as a preferred species transported (18, 21). Thus, the differences in pH sensitivity between IIa- and IIb-mediated Na/P_i cotransporter activities may be attributable to differences within the two proteins.

The aim of the present work was to identify the structural domains that confer pH sensitivity to the type IIa Na/P_i cotransporter. For this purpose, we constructed several mouse IIa-mouse IIb chimeras as well as mutated Na/P_i cotransporters and compared their pH dependences with those of the wild type proteins. These studies were done by expression of the various constructs in *X. laevis* oocytes and measurement of the Na^+-dependent P_i uptake over an extracellular pH range from 6.2 to 8. Our data suggest that the third extracellular loop is involved in conferring the pH sensitivity of the IIa-mediated P_i transport. Within this loop, a cluster of three charged amino acids (REK), present in IIa but not in IIb Na/P_i cotransporters, are necessary to provide the pH dependence to IIa.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotide primers were obtained from Microsynth (Balgach, Switzerland). The site-directed mutagenesis kit containing *Pfu* DNA polymerase was purchased from Stratagene, and the restriction and modifying enzymes were from Amersham Pharmacia Biotech or Life Technologies, Inc. All chemicals were purchased from Fluka. All constructs were cloned in pSPORT-1 (Life Technologies, Inc.).

Construction of Chimeras (IIa/IIb)—The partial cDNA fragments used to construct the chimeras were amplified by PCR,[1] using as template the wild type (WT) mouse IIa or IIb cDNAs subcloned into pSPORT and the indicated nucleotides as primers (see Table I). PCR reactions were performed using *Pfu* DNA Polymerase, and 30 thermal cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min/kb of PCR target. The different chimera constructs used in this study are schematized in Fig. 1.

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[1] The abbreviations used are: PCR, polymerase chain reaction; WT, wild type; kb, kilobase; TD, transmembrane domain.

TABLE I
PCR primers sequence used to make several constructs

Name	Primer
1-*Sph*I	5′–GAAAGCTGGTAC**GCATG**CAGGTACCGGTCC–3′
2-*Bgl*II	5′–GTCTGGGTGACAC**AGATCT**GAATGAGACTGTG–3′
3-*Sal*I	5′–GAGCTATGAC**GTCGAC**TGCACGCGTACG–3′
4-*Bgl*II	5′–CACAGTCTGATTC**AGATCT**GGTGTCACCCAG–3′
5-*Nhe*I	5′–CCAGGCATAACACAA**GCTAGC**TCTTGTGATAAGGCC–3′
6-*Nhe*I	5′–GCCCTTATCACAAGA**GCTAGC**TTGTGTGTTATGCCAGG–3′
7	5′–GAGCTATGACGTCGCATGCACGCGTACG–3′
8-*Nhe*I	5′–CTGGAAGCAGGT**GCTAGC**CAGGGAGATGAC–3′
9	5′–GAAAGCTGGTACGCTTGCAGGTACCG–3′
10*Nhe*I	5′–GTCATCTCCCTG**GCTAGC**ACCTGCTTCCAG–3′
11-*Nhe*I	5′–GACAAGAAAGGTCAT**GCTAGC**ACCCACCAC–3′
12-*Nhe*I	5′–GCAGGGCAGCG**GCTAGC**ACAGTAGGATGCC–3′
13-*Nhe*I	5′–CTTGTGGGG**GCTAGC**ATGACCTTC–3′
14-*Nhe*I	5′–GGAATATTGCTTT**GCTACGC**CAATCCCATTC–3′
GNT-s	5′–GCCATCCTGGCAGCCGTTGCCAGCCCC**GCCAACACC**CTATCCAGCTCATTTCAGATTGCCCTC–3′
GNT-as	5′–GAGGGCCAATCTAAATGAGCTGGATAG**GGTGTTGGC**GGGGCTGGCAACGGCTGCCAGCATGCC–3′
REK-s	5′–GCCATTCTGGCTGCTTTAGCCAGCCCA**AGGGAGAAG**TTGAGGAGTTCTCTCCAGATTGCCCTG–3′
REK-as	5′–CAGGGCAATCTGGAGAGAACTCCTCAA**CTTCTCCCT**TGGGCTGGCTAAAGCAGCCAGAATGGC–3′

The IIa-IIb chimera contained the N-terminal cytoplasmic tail plus the first three transmembrane domains (TDs) of the IIa and the last five TDs plus the C-terminal cytoplasmic tail of IIb (Fig. 1). To obtain the IIa fragment, WT IIa was amplified using the primers 1-*Sph*I and 2-*Bgl*II in which *Sph*I and *Bgl*II restriction sites were introduced (shown in bold in Table I). Then, the PCR fragments as well as the WT IIb were digested with *Bgl*II and *Sph*I. The double digestion of the WT IIb cotransporter produced two fragments: one of about 1 kb containing the N-terminal portion, and one other of about 7 kb containing the last five TDs plus the C-terminal cytoplasmic tail and the whole pSPORT sequence. After purification in 1% agarose gel, the appropriate fragments were ligated over night with T4 Ligase at 16 °C.

The IIb-IIa chimera contained the N-terminal cytoplasmic tail plus the first three TDs of IIb, and the last five TDs plus the C-terminal cytoplasmic tail of IIa (see Fig. 1). The IIa fragment was amplified using the primers 3-*Sal*I and 4-*Bgl*II in which the indicated restriction sites were introduced (shown in bold in Table I). Then, the WT IIb and the IIa-PCR product were digested with *Sal*I and *Bgl*II. This double digestion released two fragments from the WT IIb cotransporter: one of about 4.5 kb containing the three N-terminal TDs and the whole pSPORT sequence, and another of about 3.5 kb containing the C-terminal portion. After purification in agarose gel, the appropriate fragments (see Fig. 1) were ligated as described before.

The IIaN-IIbC chimera contained the N-terminal tail plus the eight TDs from IIa and only the C-terminal cytoplasmic tail from IIb cotransporter (see Fig. 1). We used the primers 1-*Sph*I and 5-*Nhe*I to amplify the IIa fragment and primers 10-*NheI* and 9 (downstream of a *Sal*I site located in the polylinker) and 10-*Nhe*I to amplify the C-terminal tail of the IIb. Both PCR fragments were digested with the corresponding restriction enzymes. In addition, pSPORT was digested with *Sph*I and *Sal*I. After purification in agarose gel, a three-fragments ligation was carried out overnight.

The IIbN-IIaC chimera contained the N-terminal tail plus the eight TDs of IIb and only the C-terminal cytoplasmic tail of IIa (see Fig. 1). We amplified by PCR the IIb fragment using the primers 7 (upstream of a *Sph*I site located in the polylinker) and 8-*Nhe*I; for amplification of the IIa cytoplasmic tail, the primers 3-*Sal*I and 6-*Nhe*I were used. Then, we followed a strategy similar to that described for the IIaN-IIbC chimera.

The IIaN6-IIb chimera contained the N-terminal tail plus the first six TDs from IIa and the last two C-terminal TDs plus the C-terminal tail from IIb cotransporter (see Fig. 1). The IIa fragment was amplified using primers 1-*Sph*I and 12-*Nhe*I and the IIb portion with primers 14-*Nhe*I and 9. We then followed an approach similar to that described for the IIaN-IIbC chimera.

Plasmids encoding the chimeric constructs were introduced into *Escherichia coli* competent cells. After plasmid purification sequences were verified by automatic sequencing (Microsynth, Balgach, Switzerland).

Construction of IIa and IIb Point Mutations—Mutations of the REK residues of IIa to GNT, and GNT of IIb to REK, were done by site-directed mutagenesis. Briefly 20 ng of the plasmids containing the WT mouse IIa or mouse IIb cDNAs were amplified with 2.5 units of *Pfu* DNA polymerase in the presence of 250 nM overleaping primers containing in the middle of their sequence the three mutated codons

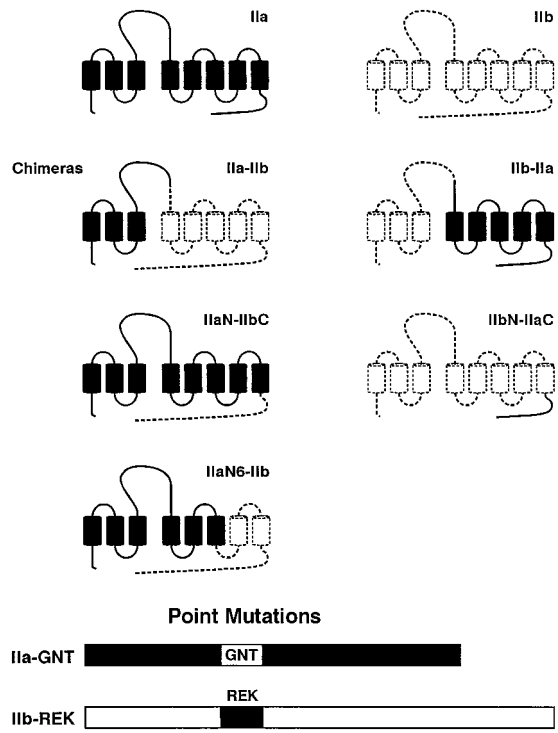

FIG. 1. **Schematic representation of the wild type (*WT*) chimeric constructions and mutations of IIa and IIb proteins**.

(GNT-s and GNT-as for IIa, and REK-s and REK-as for IIb; see Table I). PCR amplification was performed with 25 thermal cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 16 min. Then, 10 units of *Dpn*I were added directly to the amplification reaction, and the sample was incubated for 1 h at 37 °C to digest the parental, methylated DNA. XL1-blue supercompetent cells were finally transformed with 1 μl of the reaction mixture and plated onto LB-ampicillin plates. After plasmid purification sequences were verified by automatic sequencing (Microsynth, Balgach, Switzerland).

X. laevis Oocyte Expression and Transport Assay—The procedures for oocytes preparation and cRNA injection, as well as for the $^{32}P_i$ uptake assay have been described in detail elsewhere (22). Briefly, the cDNAs encoding the two WT and the constructs were first linearized. *In vitro* synthesis and capping of cRNAs were done by incubating the linearized cDNAs in the presence of Cap Analog (New England Biolabs, Inc.) and 40 units of T7 RNA polymerase (for WT IIa and IIa-GNT) or Sp6 RNA polymerase (for WT IIb, IIb-REK, and the chimeric constructs). Oocytes were injected with either 50 nl of water or 50 nl of water containing 5 ng of cRNA. $^{32}P_i$ uptake was measured 4 days after cRNA injection as already described (22), using external solutions with pH adjusted to 6.2, 7.4, and 8.

Throughout the study, we have shown experiments obtained with single batches of oocytes, with 8–10 oocytes measured for each experimental condition. All studies have been performed with qualitatively similar data on at least three batches of oocytes.

RESULTS AND DISCUSSION

Mouse Type IIa-Type IIb Chimeras—To identify the regions responsible for the different pH dependence of the IIa and IIb Na/P_i cotransporters, we have constructed several chimeras (Fig. 1). The first set of constructs contained approximately half of each type of cotransporter: the IIa-IIb chimera comprised the first 304 amino acids (N-terminal cytoplasmic tail and the three first TDs) from IIa, and the last 395 amino acids (last five TDs and the C-terminal cytoplasmic tail) from IIb. In the complementary IIb-IIa chimera, the first 301 amino acids correspond to IIb and the last 332 amino acids to the IIa. Because most of the differences between IIa and IIb reside in the C-terminal cytoplasmic tails, we constructed a second set of chimeras with only interchanged cytoplasmic tails. Thus, the IIaN-IIbC chimera comprised the first 596 amino acids (N-terminal cytoplasmic tail plus eight TDs) of IIa and the last 84 amino acids (C-terminal tail) from IIb. To obtain the complementary IIbN-IIaC chimera, we fused the first 612 residues of IIb to the last 41 amino acids of IIa. Finally, the IIaN6-IIb chimera was constructed by fusing the first 487 residues (N-terminal cytoplasmic tail plus six TDs) of IIa to the last 193 amino acids (last two TDs plus the C-terminal tail) of IIb.

Based on Western blot analysis using IIa- and IIb-specific antibodies (11, 12), we found that all chimeras were synthesized in oocytes after cRNA-injection although their level of expression was only about 25–30% compared with the wild types (data not shown). In a previous study we have shown that, in oocytes, transport activity correlates with the transporter present in the surface membrane but not with the total amount of protein (23). Here, we have measured Na^+-dependent P_i uptake and its pH dependence for all the different constructs. Because we did not quantify the transporter at the surface, we cannot make conclusions about the influence of the different chimeric constructions on overall transport characteristics (*e.g.* transport rates) but only on the effect on pH dependence.

As shown in Fig. 2, the P_i transport activities of all chimeric constructions were significantly higher than that of water-injected oocytes in the presence of 100 mM Na^+ and at pH 7.4. The P_i uptake activities were entirely dependent on the presence of Na^+ (data not shown). Thus, all chimeric proteins are present at the oocyte surface and mediate Na/P_i cotransport. This was a prerequisite for our analysis of the pH dependence of P_i transport activity mediated by these chimeric proteins (see below).

The pH dependence of the WT IIa and IIb as well as the chimeric cotransporters was analyzed in oocytes by measuring the Na^+-dependent $^{32}P_i$ uptake at three external pH values: 6.2, 7.4, and 8. As shown in Fig. 3*A*, the Na/P_i cotransport mediated by the WT IIa increased with higher pH values. This increase is a characteristic feature of IIa cotransporters (7) that most likely reflects an interaction of protons with the unloaded carrier and the last Na^+ binding step.[2] In contrast, IIb-mediated Na/P_i cotransport was largely independent of the external pH and was even slightly higher at more acidic pH (11).

As shown in Fig. 3*B*, the IIb-IIa chimera behaved similarly to the WT IIa, with higher activity at higher pH values. In contrast, the IIa-IIb chimeras showed no pH dependence, similarly to the WT IIb. Therefore, the pH dependence can be attributed to sequences within the C-terminal half of IIa. We next analyzed the chimeras in which only the C-tails were interchanged. As shown in Fig. 3*C*, the C-terminal tail does not seem to be involved in the pH dependence of IIa since replacement with the IIb tail did not abolish the pH dependence of IIa, and conversely the IIa tail did not restore the pH dependence of the

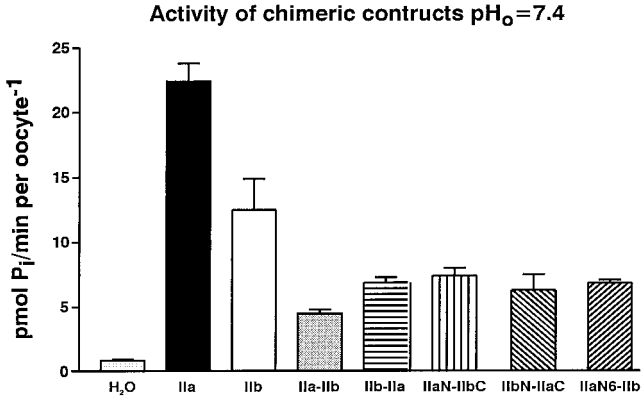

FIG. 2. **P_i uptake of WT and chimeric constructions.** Oocytes injected either with water or cRNA (50 ng per oocyte) corresponding to WT (IIa, IIb) and different chimeras (IIa-IIb, IIb-IIa, IIaN-IIbC, IIbN-IIaC, IIaN6-IIb) were assayed after 4 days for P_i uptake (at 0.5 mM P_i) in 100 mM Na, at pH 7.4. The *bars* represent the mean ± S.E. obtained from eight to ten oocytes per group of a representative experiment (n = 3).

[2] I. Forster, J. Biber, and H. Murer, submitted for publication.

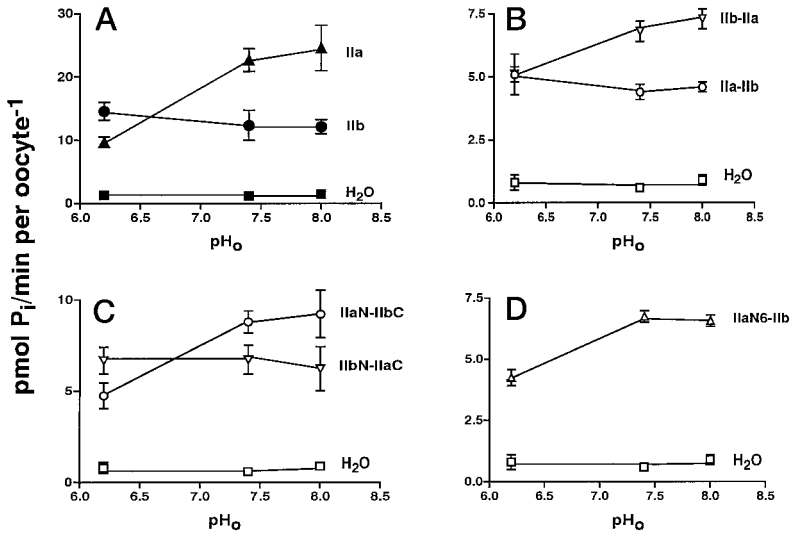

FIG. 3. **pH dependence of P_i uptake mediated by WT and chimeric constructs of type II Na/P_i cotransporters.** *A*,WT IIa and IIb; *B*, IIa-IIb and IIb-IIa chimera; *C*, IIaN-IIbC and IIbN-IIaC chimera; *D*, IIaN6-IIb chimera. P_i uptake was measured in the presence of 100 mM Na^+ and buffers with pH 6.2, 7.4, or 8. Each *point* represents means ± S.E. of eight to ten oocytes per group of a representative experiment (n = 3).

```
                                                  **********
mouseIIa    QSSSVFTSAITPLIGLGVISIERAYPLTLDSNIGTTTTAILAAVASPREKLSSSFQ
mouseIIb    QSSSVFTSAMTPLIGIGVISIERAYPLTLGSNIGTTTTAILAALASPGNTLPSSIQ

Consensus   QSSSVFTSA.TPLIG.GVISIERAYPLTL-SNIGTTTTAILAA.ASP-.-L-SS.Q
```

FIG. 4. **Sequence comparison of the third extracellular loop of IIa and IIb.** Charged residues not conserved between IIa and IIb are shown in *bold*. *Period*, conserved changes; *hyphen*, non-conserved changes; *asterisk*, cysteine scanning.[2]

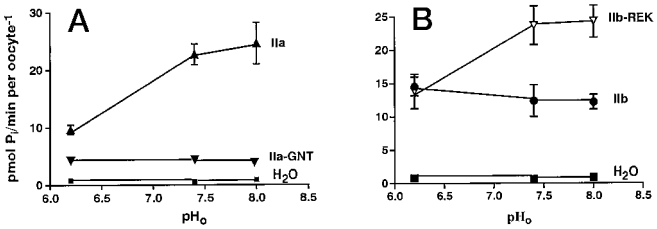

FIG. 5. **Site-directed mutagenesis in the third extracellular loop.** *A*, pH dependence of WT IIa and IIa-GNT mutant. *B*, pH dependence of WT IIb compared with IIb-REK construct. We used the same conditions as described in Fig. 3.

IIb. These experiments suggested that the pH-dependent site is located within the last five TDs of IIa. To locate more specifically the pH-sensitive region, we tested a chimera containing the first six TDs of IIa and only the last two TDs and the C-terminal tail of IIb. Fig. 3*D* shows that this chimera (IIaN6-IIb) has pH sensitivity similar to the WT IIa. Thus, the domain responsible for the pH sensitivity of IIa is located between the fourth and sixth TD, *i.e.* within the third extracellular loop.

Site-directed Mutagenesis in the Third Extracellular Loop—In a cysteine-scanning study on to the rat IIa cotransporter, the third extracellular loop was found to be of functional importance (25). A sequence comparison of this loop revealed a high degree of homology between the mouse IIa and IIb; however, a cluster and single residues differ (Fig. 4). Among them, three charged amino acids (REK) present in IIa correspond to three neutral amino acids (GNT) in IIb. Single amino acid substitution in the rat IIa cotransporter indicated that replacement of charged amino acid residues at sites 462 (R) and 464 (K) by cysteines, reduced the pH sensitivity of the mutants as compared with the WT.[3] Moreover, these charged amino acids are conserved in IIa proteins identified from various species, all of which show the same characteristic increase in transport activity with increasing pH (7, 11). Therefore, we investigated the role of these three residues in determining pH sensitivity of IIa. Replacement of the charged amino acids in the IIa protein by the IIb neutral residues (construct IIa-GNT) led to the loss of the characteristic IIa pH dependence, such that the cotransporter behaved similarly to the WT IIb (Fig. 5, *A* and *B*). Furthermore, pH sensitivity could be introduced into the IIb protein by replacing the neutral residues with the IIa charged amino acids residues (IIb-REK) so that this construct now mimicked the strong pH dependence of the WT IIa (Fig. 5, *A* and *B*). The behavior of these two constructs strongly suggested that the cluster of three charged amino acids (REK) located on the third extracellular loop is responsible for the pH dependence of IIa.

The role of specific domains and charged amino acids in conferring pH sensitivity has been described for other transport proteins. For example, for the erythrocyte K-Cl-cotransporter, internal histidine residues are involved in the inhibition at acidic pH (26). A charged residue (Glu-419) has been reported to play a role in the pH sensitivity of a chloride channel (ClC-2G419) (27). For the bacterial Na^+/H^+ antiporter (NhaA), His-225 has been proposed as part of the pH sensor (28); furthermore, Leu-73 and/or its vicinity may contribute to the pH sensitivity of the antiporter (29). In the mammalian Na^+/H^+ antiporter (NHE1 and related isoforms), a pH-modifier site seems to be located in the N-terminal TD although its precise sequence has not been identified (30). Also for the mammalian AE2 anion exchanger, a chimera approach led to the identification of the region involved in the pH sensitivity of anion exchanger (24).

In conclusion, we have identified a "molecular determinant" of the pH sensitivity of the IIa Na/P_i cotransporter, namely three charged amino acids located in the third extracellular loop (REK) which play a critical role in determining the specific pH dependence of the renal cotransporter.

[3] G. Lambert, G. Stange, I. Forster, J. Biber, and H. Murer, unpublished observations.

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